Protein Science (1994), 3:711-712. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Charge reversal at the P3′ position in protein C optimally enhances thrombin affinity and activation rate

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(RECEIVED January 19, 1994; ACCEPTED February 1, 1994)

Abstract: We have examined the properties of several human protein C (HPC) derivatives with substitutions for acidic residues near the thrombin cleavage site, including changing the P3′ Asp to Asn (D172N), Gly (D172G), Ala (D172A), or Lys (D172K). The rate of thrombin-catalyzed activation of D172N, D172G, and D172A was increased 4-9-fold compared to wild-type HPC, primarily due to a reduction in the inhibitory effect of calcium and a resulting increase in affinity for free α-thrombin. There was no significant increase in activation rate or affinity with these 3 derivatives in the absence of calcium, confirming that P3′ Asp affects calcium dependency in the native protein C molecule. With charge reversal at P3′ (D172K), there was a 30-fold increase in activation rate in the presence of calcium, but unlike the other derivatives, there was a substantial effect (5-fold) on the activation rate and affinity for free α-thrombin in the absence of calcium. Thus, protein C affinity for thrombin appears to be influenced by a combination of calcium-dependent and -independent effects of the acidic P3′ residue.

Keywords: coagulation; protein C; serine protease; thrombin

Human protein C (HPC) is a plasma serine protease that when activated by thrombin serves as a major feedback regulator of the coagulation cascade (reviewed by Esmon, 1987). At physiological levels of calcium, protein C is a very poor substrate for free α-thrombin, however, it is a relatively good substrate for thrombin in the absence of calcium, or when thrombin is complexed with the integral membrane protein, thrombomodulin. Previously, we and others have shown by site-directed mutagenesis that neutralization of acidic residues near the thrombin cleavage site e.g., Asp at the P3 and P3′ positions, increases activation by free α-thrombin due to a reduction in the inhibitory effect of calcium (Ehrlich et al., 1990; Rezaie & Esmon, 1992; Richardson et al., 1992). When bound to thrombomodulin, the inhibitory influence of these acidic residues in protein C has been suggested to be reduced due to a conformational change in thrombin (Ye et al., 1991). In this report, we demonstrate that the acidic P3′ residue not only affects calcium-induced inhibition, but that the charge at this position plays a major role in the poor affinity of protein C for thrombin.

Shown in Figure 1A is a schematic of the native HPC molecule, and the location of the P3′ Asp residue that was changed to Asn (D172N), Gly (D172G), Ala (D172A), or Lys (D172K). There was no significant effect of these alterations at P3′ on the functional protease activity as determined by previously described methods (Grinnell et al., 1991). Each of the substitutions for the acidic residue at P3′ resulted in an increase in the rate of activation in the presence of calcium (Fig. 1B). The increase in rate appeared to be related to the polarity of the change, with the reversal of charge from acidic (wild type) to basic (D172K) having the greatest effect on rate, ~30-fold. As expected, the inhibitory effect of calcium on α-thrombin-catalyzed activation was reduced, with half-maximal inhibitory calcium concentrations for each derivative giving similar values in the range of 0.4-0.8 mM, compared to ~0.15 mM for the wild type. The magnitude of this effect on calcium inhibition was not, however, as great as the value of 4.4 mM reported with the D172G substitution in protein C lacking the Gla-domain (Rezaie & Esmon, 1992), suggesting that the Gla-domain attenuates the calcium dependency of thrombin activation.

Although the D172K change did not appear to reduce the calcium inhibition substantially more than that of the other derivatives, the rate of activation of D172K was substantially higher. We determined activation rates in the absence of calcium (5 mM EDTA), and, in contrast to the results above, derivatives D172N, D172G, and D172A showed no significant increase in the rate of α-thrombin-catalyzed activation, but D172K still showed a 5-fold increase. Kinetic analyses showed increased affinity of D172K for thrombin as demonstrated by a decrease in K_m from ~24 μM for wild-type HPC to ~5 μM for D172K, with little change in k_{cat}. Thus, there appears to be a direct affect of the acidic P3′ residue on affinity for thrombin that is independent of the previously described effect of this residue on calcium-dependent activation. Based on structural analysis of thrombin, it can be speculated that the Glu at position 39 in the S3′ sub-

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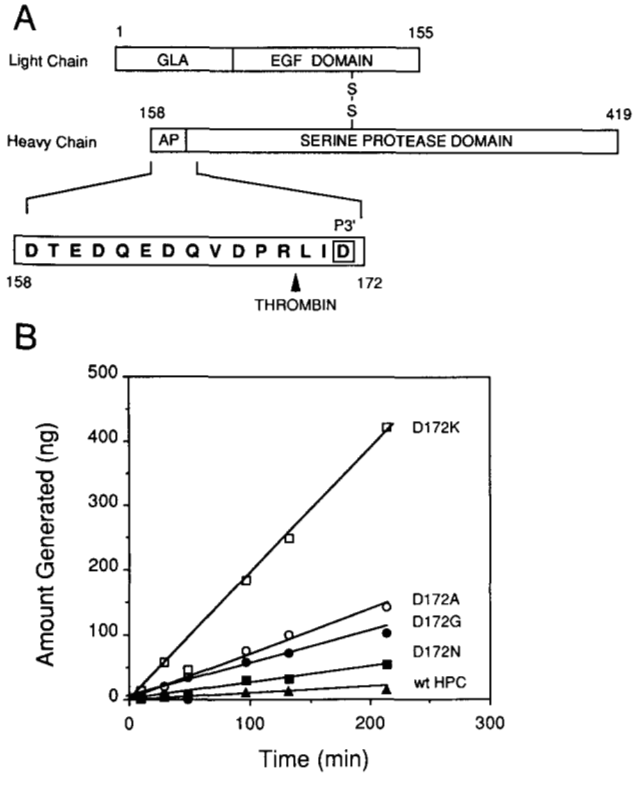

Fig. 1. Analysis of thrombin-catalyzed activation rates of protein C derivatives with substitutions at the P3′ residue. **A:** Schematic representation of processed protein C. Numbers refer to amino acid positions in the mature protein (following removal of the signal sequence and propeptide). The light chain contains a region of γ-carboxyglutamic acid (GLA) residues that is required for calcium-dependent membrane binding and functional activity (reviewed by Gardner & Griffin, 1983), and 2 epidermal growth factor (EGF)-like regions. The site for thrombin cleavage of the activation peptide (AP) is indicated. The designation P3′ refers to the third position C-terminal to the thrombin cleavage site. Each amino acid change was made by PCR site-directed mutagenesis of the cDNA coding sequence using as primers: D172A, 5′-TCT CTC CCG CGG CTC ATT **GCT** GGG AAG ATG A-3′; D172G, 5′-TCT CTC CCG CGG CTC ATT **GGT** GGG AAG ATG A-3′; D172K, 5′-TCT CTC CCG CGG CTC ATT **AAG** GGG AAG ATGA-3′. Reconstruction of the coding sequence, the primer for D172N, and expression vector construction were essentially as described previously (Richardson et al., 1992). Stable recombinant 293 cell lines secreting wild-type HPC and each of the derivatives were created by isolation of hygromycin-resistant clones as previously described (Grinnell et al., 1991), and HPC in the conditioned culture medium was purified from serum-free conditioned culture medium by published procedures (Yan et al., 1990). **B:** Activation rates were determined at 37 °C using human α-thrombin as described previously (Grinnell et al., 1991). The amount of activated protein C generated was less than 10% of the initial zymogen in all experiments.

site of thrombin could interact with the P3′ residue of thrombin substrates (reviewed by Stubbs & Bode, 1993). Of interest, LeBonniec and Esmon (1991) generated a mutant of thrombin E39K that activated protein C several-fold faster, and it was suggested that this change in thrombin mimicked the catalytic switch induced by thrombomodulin. Our data would be consistent with charge repulsion between the P3′ Asp of protein C and Glu 39 of thrombin, resulting in reduced affinity, and conversely the increased activation rate observed with P3′ mutant D172K could be due to a more favorable charge interaction at this thrombin subsite. Our data would also favor a direct electrostatic repulsion in the substrate binding pocket independent of conformational change. Overall, protein C affinity for thrombin appears to be influenced by a combination of calcium-dependent and -independent effects of the acidic P3′ residue.

Of further interest, we combined the previously described change at the P3 position, D167F (Ehrlich et al., 1990), with each P3′ derivative, and the resulting combination resulted in a synergistic increase in activation rate. Strikingly, D167F/D172K showed an activation rate approximately 100-fold higher than wild-type HPC in the presence of calcium, again due to a combination of decreased calcium inhibition and increased affinity observed even in the absence of calcium (data not shown). These data differ from the results of Rezaie and Esmon (1992) using Gla-domainless protein C, in which acidic P3 plus P3′ neutralization did not result in synergy, further suggesting that the Gla-domain influences calcium-dependent activation, despite the demonstration of Gla and epidermal growth factor-independent calcium sites for activation (Rezaie & Esmon, 1992; Rezaie et al., 1992).

Acknowledgments

The assistance of Bruce Glover in the synthesis of oligonucleotides and of Stan Burgett and Ivan Jenkins in DNA sequencing is acknowledged.

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